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Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Acidic Glycoconjugates Facilitated by the Use of Spermine as a Co-matrix

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Negative-ion matrix-assisted laser desorption/ionization mass spectra of sialylated glycoconjugates were acquired employing 2,5-dihydroxybenzoic acid (DHB) in conjunction with spermine as a co-matrix. The addition of spermine to DHB permitted an improved crystal formation as well as a higher analyte solubility. Moreover, DHB/spermine appears to minimize alkali adduct formation, thus allowing the sample analysis without desalting. The combined matrix permitted the analysis of complex sialylated and sialylated/fucosylated structures down to the femtomole range. The ability to use such a matrix also facilitates determination of the sialic acid linkages (in combination with a specific enzyme cleavage). The matrix also appears suitable for studies on gangliosides. (J Am Soc Mass Spectrom 1998, 9, 1293–1302) © 1998 American Society for Mass Spectrometry

An increasing need to understand the numerous functions of glycoconjugates in biological systems has stimulated intensive search for new strategies toward structural elucidation of carbohydrates. During the most recent period, mass-spectrometric (MS) techniques have been increasingly applied to characterize glycan moieties in various biologically important molecules. Mass spectrometry offers distinct advantages over the more conventional approaches because of its inherent sensitivity and, potentially, its capability to solve complex structures through tandem (MS/MS) techniques.

The power of MS/MS techniques in structural elucidation of complex oligosaccharides has particularly been demonstrated through the use of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The appealing features of this approach were shown through an in-source decay with a magnetic sector instrument [1], postsource decay on a reflectron time-of-flight (TOF) instrument [1–3] and collision-induced dissociation (CID) on either a magnetic sector instrument fitted with an orthogonal TOF analyzer [1], or an external-source Fourier-transform mass spectrometer adopting a MALDI source [4]. These various approaches provide the basic means to fragment the oligosaccharide analytes, permitting a determination of sequence, branching, and the linkage forms in a studied molecule.

However, the MALDI/MS of oligosaccharides is not without problems. The presence of sialylated structures generally complicates the acquisition of analytically desirable MALDI spectra. The negatively charged sialic acid groups that often reside at the oligosaccharides' ends are easily lost during the highly energetic MALDI process [5, 6]. Also, a MALDI analysis of sialylated oligosaccharides in the positive-ion mode yields a mixture of cation adducts, thus reducing signal intensity. These problems generally account for the poorer detection limits (picomole range) of acidic oligosaccharides unless a unique matrix is used [7]. Moreover, the problems become more pronounced as the number of acidic groups present in an oligosaccharide structure increases.

In order to avoid the problems associated with the MALDI instability of acidic oligosaccharides, it is feasible to treat the sample with a suitable enzyme, such as neuraminidase, which removes the sialic acid residues. Regrettably, this procedure often destroys much useful biochemical information, as the sialic acids have often been implicated in biochemical recognition [8]. Additional approaches leading to improvements in detectability of acidic oligosaccharides in MALDI/MS include the use of matrices that have been proven effective in the analysis of oligonucleotides [7], an addition of cation-exchange resin beads to the sample solution (either directly on the probe, or prior to crystallization) [9], an esterification of sialylated structures prior to a MALDI/MS analysis [10], use of basic peptides and proteins as a co-matrix [11], or the utilization of membrane microdialysis [12]. Most, if not all, of these

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procedures may result in sample dilution or adsorptive losses.

Yet another approach demonstrated as effective in the MALDI/MS analysis of oligonucleotides involves the use of organic bases as co-matrices [13–16]. However, only some organic bases appear appropriate for the elimination of nonvolatile cation (e.g., sodium) adducts. This is because the fact that certain bases not only reduce cation adduction, but also decrease ion abundance through formation of a crystal.

We report here the use of spermine as a highly effective co-matrix to dihydroxy benzoic acid (DHB), a widely used matrix in carbohydrate analysis. The procedure reported here permits the analysis of acidic glycoconjugates as negative ions, at very low detection limits, and there is no need for cleanup or desalting procedures. The effectiveness of this sample preparation procedure has been tested here with standard acidic oligosaccharides, gangliosides, as well as the sialylated N-linked glycans released enzymatically from bovine fetuin, human α_1-acid glycoprotein (AGP) and human tranferrin.

Experimental

Materials

The standard glycoproteins (human AGP, transferrin, and bovine fetuin), N-acetylneuraminyl-lactose and N-acetylneuraminyl-lacto-N-neo-tetrose (both originated from human milk), 2,5-dihydroxybenzoic acid, gangliosides type IV from bovine brain, maltose ladder and spermine (N,N′-bis[3-aminopropyl]-1,4-butanediamine tetrahydrochloride) were obtained from Sigma Chemical (St. Louis, MO). The recombinant enzyme N-glycosidase F (PNGase F) from *E. coli* [EC 3.2.2.18] and neuraminidase from Newcastle disease virus [EC 3.2.118] were purchased from Boehringer Mannheim (Indianapolis, IN). The labeling reagent, 8-aminopyrene-2,3,4-trisulfonic acid (APTS) was received from Molecular Probes (Eugene, OR). The standard complex oligosaccharides (fucosylated, disialylated biantennary and trisialylated triantennary) were purchased from Oxford GlycoSystems (Wakefield, MA). All remaining chemicals were from Aldrich (Milwaukee, WI).

Methods

Enzymatic cleavages. The N-linked oligosaccharides were enzymatically released from their respective glycoproteins. Briefly, the glycoproteins to be digested were reconstituted in 0.025 M sodium phosphate buffer (pH 7.5), followed by the addition of PNGase F (5 mUnit/0.1 mg glycoprotein). The samples were subsequently incubated for 18 h at 37°C.

The enzymatically released oligosaccharides were recovered by applying the digestion mixtures to C_{18} Sep-Pak cartridges (Waters Corporation, Milford, MA) which had been preconditioned with methanol, acetonitrile, and aqueous methanol (1:19 v/v). The oligosaccharides were eluted with 3 mL of 10% aqueous methanol solution, lyophilized, or dried under a stream of nitrogen, and reconstituted in water to the initial glycoprotein concentration of 2 mg/mL.

Sialylated oligosaccharides were treated with neuraminidase from Newcastle disease virus by reconstituting 2.0 μL the oligosaccharide stock solution in 10 mM sodium acetate buffer (pH 5.5), followed by the addition of neuraminidase (approximately 20 mU per nmol of a substrate) and incubation for 12 h. Prior to their MALDI analysis, the samples were dried and reconstituted in 2.0 μL water.

Labeling of the maltose ladder. Maltooligosaccharides were labeled with APTS through reductive amination [17]. The maltose ladder was dissolved in 2 μL of 150 mM APTS (prepared in a 3:17 ratio of acetic acid: DMSO) and 2 μL 1.0 M sodium cyanoborohydride in water. The reaction mixture was vortexed prior to incubation in a heating block at 90°C for 2 h. Prior to its mass spectrometry analysis, the sample was drop dialyzed against deionized water for 2 h using a 1000 MWCO dialysis membrane. This APTS-labeled maltose ladder was used exclusively for MALDI calibration.

Sample and matrix preparation. The MALDI matrix was prepared by dissolving DHB crystals in 300 mM aqueous solution of spermine to obtain a saturated DHB solution. The matrix solution was then desalted by adding a few cation-exchange resin beads (NH_4^+ ionic form) to minimize the presence of sodium ions in the matrix. The samples were prepared by spotting 0.5 μL sample aliquots on a polished stainless-steel sample plate, followed by addition of 1.5 μL of the matrix solution. The spots were allowed to dry at room temperature.

Instrumentation

All mass spectra were acquired on a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm). The instrument can operate in both the reflector and linear modes and has the delayed ion-extraction capability. The linear mode was solely used in the negative ion mode. The MALDI spectra were acquired at 18 kV accelerating voltage, with delay extraction. The delay time was set to 150 ns, in addition to the instrumental delay caused by the detection electronics. A two-point external calibration was performed on the instrument prior to an analysis, utilizing the negatively charged APTS-labeled maltose ladder, achieving mass accuracy of 0.1% or better. All acquired spectra were smoothed by applying the 19-point Savitzky–Golay smoothing [18]. The MALDI instrument used in this report utilizes a multichannel plate detector and, in order to preclude the low mass ions from saturating

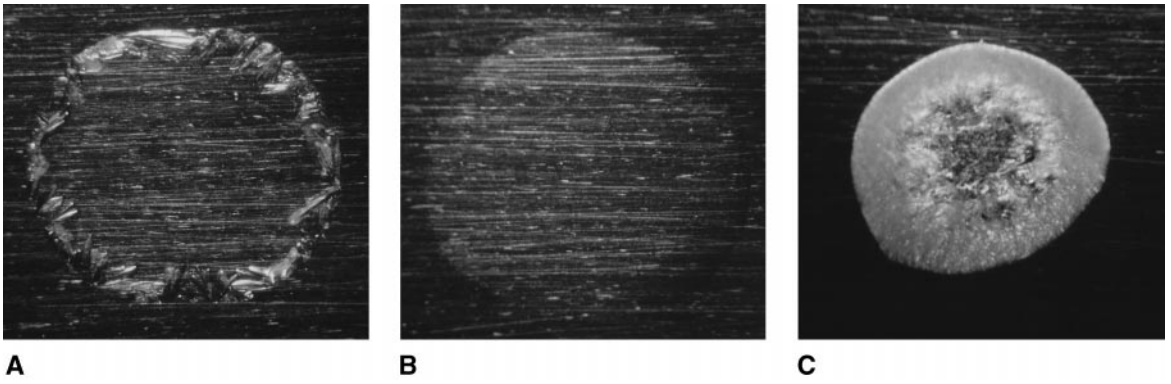

Figure 1. The effects of co-matrix on crystallization: (**a**) a spot made by mixing 0.5 μL sample with 1 μL saturated aqueous DHB solution; (**b**) a spot made by mixing 0.5 μL sample with 1 μL saturated aqueous DHB solution in 300 mM imidazole; and (**c**) a spot made by mixing 0.5 μL sample with 1 μL saturated DHB solution in 300 mM spermine.

the detector, the low-mass ion gate was set at 1000 Da in the case of N-linked oligosaccharide samples.

Results and Discussion

The MALDI matrices are supposed to absorb the laser light energy and facilitate desorption and ionization of a sample. The mixture of a sample with matrix is assumed to be vaporized from the surface of the target plate upon irradiation with the laser pulses [19]. Moreover, the irradiation may also catalyze a chemical conversion, leading to either protonated or alkaliated molecules in the positive ion mode, or deprotonated molecules in the negative ion mode. Although the details of ionization process are somewhat nebulous, it would appear that photochemical products from the matrix are responsible for the ionization [19]. It is further believed that a close contact between the matrix and the sample is essential for the formation of sample ions. A cocrystallization of the matrix and sample may aid accomplishing this.

In the case of DHB matrix, crystallization tends to occur at the periphery of the target spot in the form of long needles pointing toward the center of the target (Figure 1a). Such crystallization is believed to be responsible for a weak signal observed under such conditions. Some improvements are expected through recrystallization of the spotted material with ethanol [20]. Karas et al. [21] also succeeded in incorporating certain additives into the DHB matrix and achieved crystallization in more even layers which were observed to improve MALDI analysis of neutral oligosaccharides.

To facilitate the use of DHB as a matrix for the analysis of acidic oligosaccharides, we have experimented with the application of various organic bases as the additives which could minimize, or even eliminate, cation adduction. Encouraged by an earlier success [22] with correlation between microcrystallinity of neutral arabinosazone and its desirable MALDI behavior vis-à-vis neutral oligosaccharides, we sought a uniform crystal appearance while experimenting with different organic base additives. Some initial experiments with imidazole were only marginally successful, presumably because of the fact that only a translucent spot was obtained after drying the mixture of sample and matrix under any imidazole concentration (Figure 1b). Spermine proved to be a more useful additive, permitting a formation of well defined crystals (Figure 1c). At its optimum concentration of 300 mM, spermine in a co-matrix with DHB facilitated a highly desirable MALDI behavior for various acidic oligosaccharides, as demonstrated on several examples below.

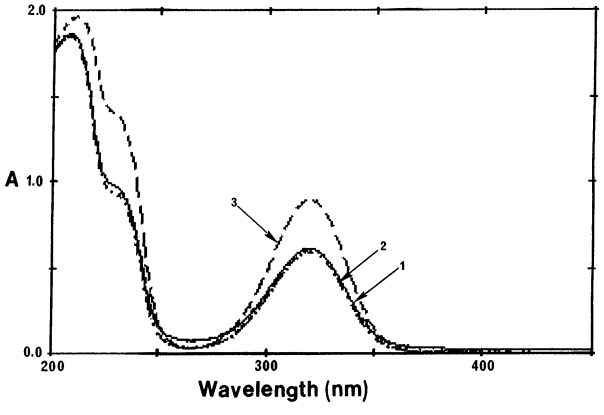

Figure 2. UV spectra of saturated DHB aqueous solution (1), 300 mM spermine prepared in saturated DHB aqueous solution (2), and saturated DHB solution prepared in 300 mM spermine aqueous solution (3).

In order to determine the additive effect of spermine on spectroscopic properties of DHB, UV absorption spectra of saturated DHB solutions were compared in the presence and absence of 300 mM spermine (Figure 2). Apparently, no major spectral shifts were observed following the inclusion of spermine, although the solution absorbance increased. This is presumably because of the higher solubility of DHB in the spermine aqueous solution.

It has been observed in this laboratory that sensitiv-

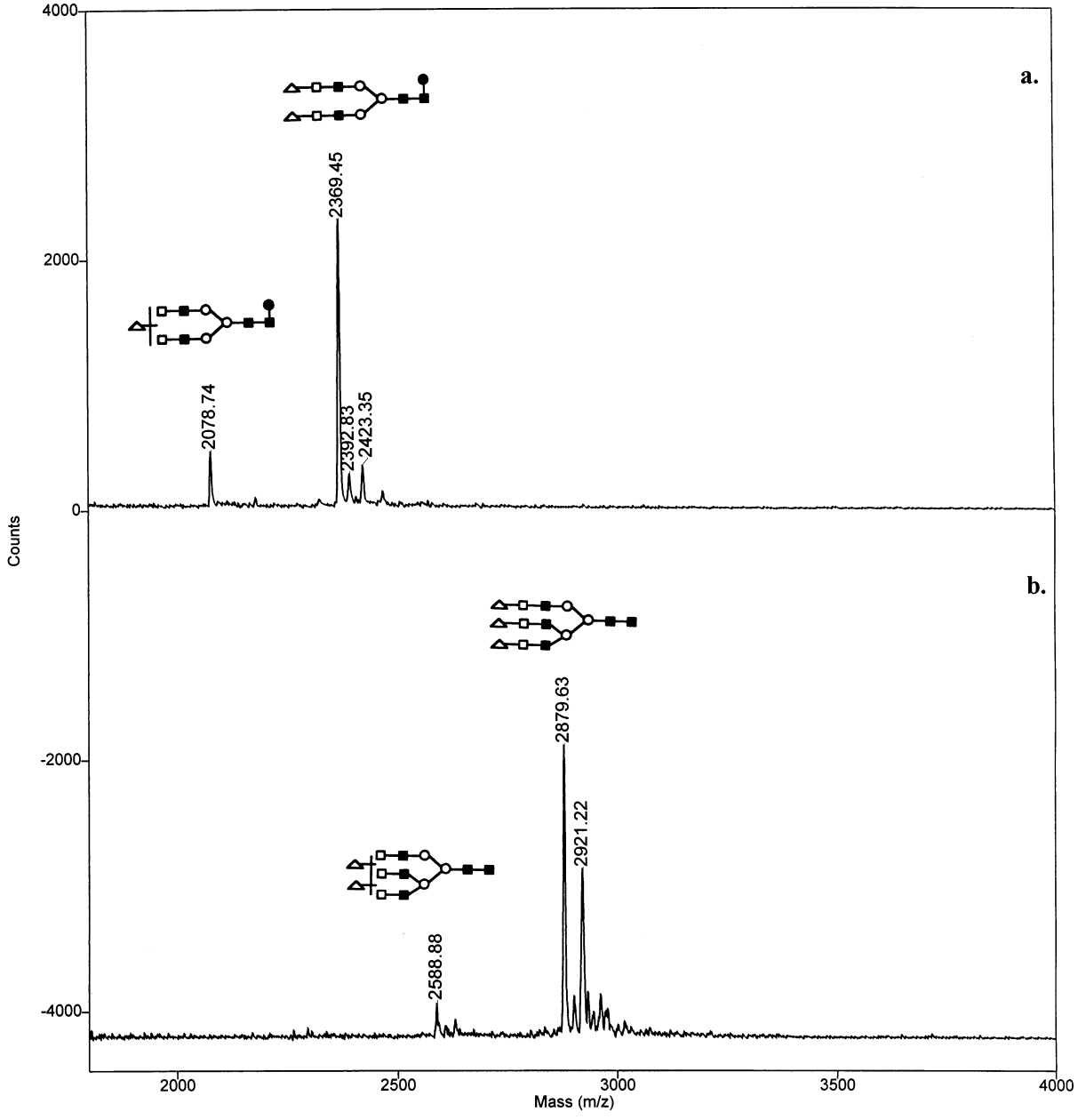

Figure 3. Negative-ion spectra of complex oligosaccharides: 50 fmol A2F (**a**) and 50 fmol A3 (**b**). Symbols: open circle = mannose, filled square = N-acetylglucosamine, open square = galactose, filled circle = fucose, and open triangle = N-acetylneuraminic acid.

ity of MALDI/MS in the analysis of acidic oligosaccharides using DHB as a matrix is at least an order of magnitude less than for the neutral oligosaccharides. To assess the improvement in sensitivity after the inclusion of spermine as a co-matrix, four standard glycans were utilized: N-acetylneuramine-lactose (NeuAc-Lac) and N-acetylneuramine-lacto-N-*neo*-tetrose (NeuAc-LacN), both isolated from human milk; a complex-type disialylated biantennary, N-linked oligosaccharide with a core fucose (A2F); and a complex-type trisialylated, galactosylated triantennary N-linked oligosaccharide (A3). The human milk glycans gave clearly the expected negative-ion signals (m/z 631.02 for NeuAcα2-3/6 Galβ1-4Glc, and m/z 997.77 for NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc) at 50 fmol level. Equally successful were the runs with A2F and A3 oligosaccharides, with their mass spectra illustrated in Figure 3a, b. In the case of A2F, a major signal is observed at m/z 2369, corresponding to $[M - H]^-$. Four additional signals were detected at m/z 2078, 2392, 2423, and 2469. The value at 2078 corresponds to the parent structure minus one sialic acid group. However, the occurrence of this signal is unlikely to be because of a facile fragmentation of A2F, but rather a contamination

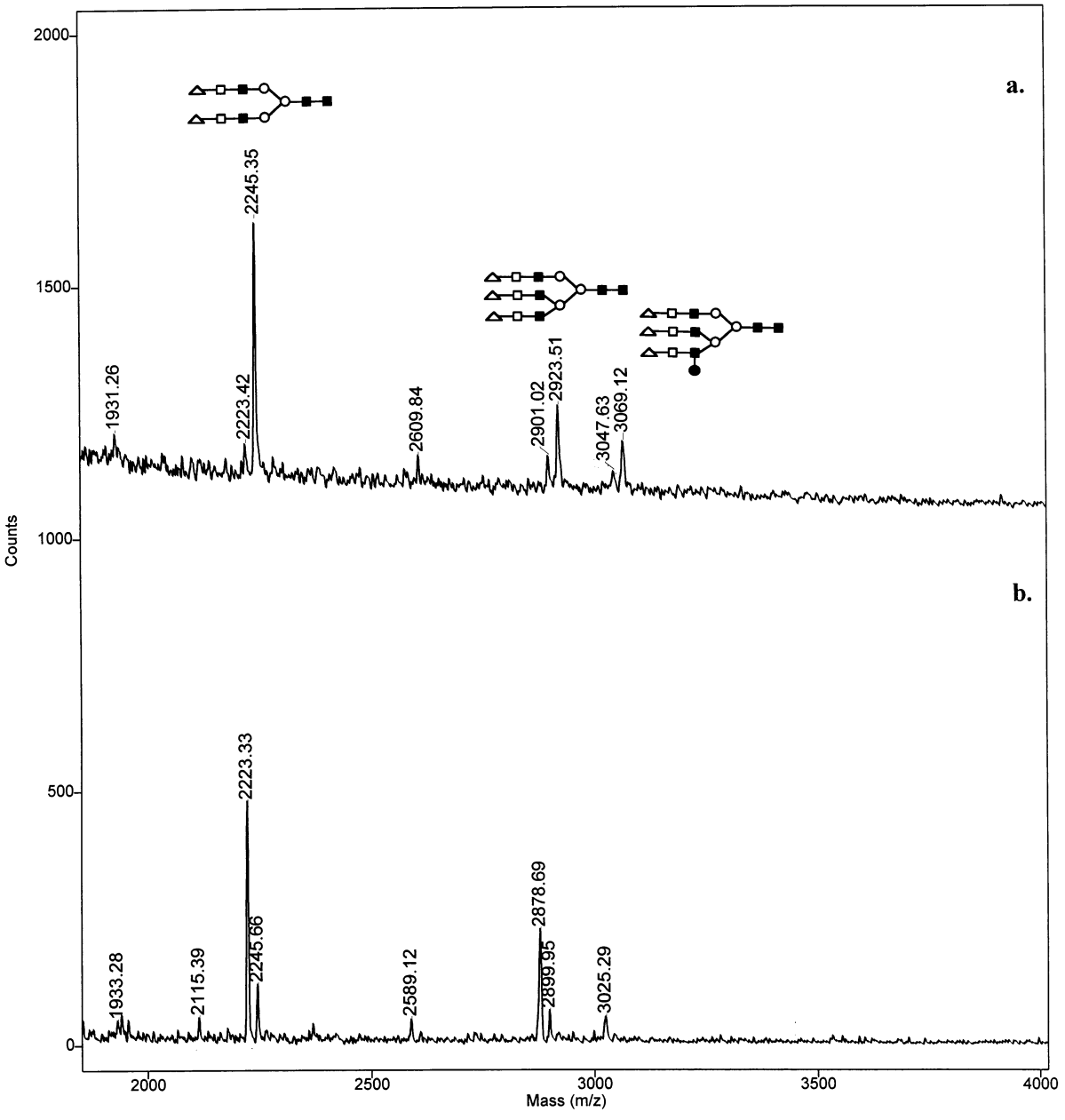

Figure 4. Negative-ion spectra of the N-linked oligosaccharides derived from the equivalent to 1 μg human AGP before (**a**) and after (**b**) drop dialysis. Symbols as in Figure 3.

in the standard: a chromatogram obtained through the pulsed-amperometric detection (provided by the supplier) showed clearly a peak corresponding to a monosialylated structure. Contamination is also believed to be responsible for m/z 2423 and 2469 (as consistent with the manufacturer's chromatographic data), whereas m/z 2392 (Figure 3a) corresponds to $[M+Na-2H]^-$.

In the spectrum of A3 (Figure 3b), two prominent peaks appear at m/z 2879 and 2921, corresponding to $[M-H]^-$ ion and an O-acetylated A3 ($[M+CH_2CO-H]^-$), respectively. The very weak signal at m/z 2588 seems to correspond to a loss of one sialic acid residue. Once again, the manufacturer's chromatogram implicated these as impurities. It should be noted that the standard oligosaccharides were prepared in pure aqueous solutions, so that the major signals observed for A2F and A3 were the deprotonated ions. However, when the standards were prepared in solution at a moderate buffer concentration (e.g., in 50 mM phosphate buffer), sensitivity was not drastically reduced, with the major ions appearing as sodium adducts (data not shown).

Although the above data on standard oligosaccharides were essential to establish the detection attributes of negative-ion MALDI/MS using spermine as a co-

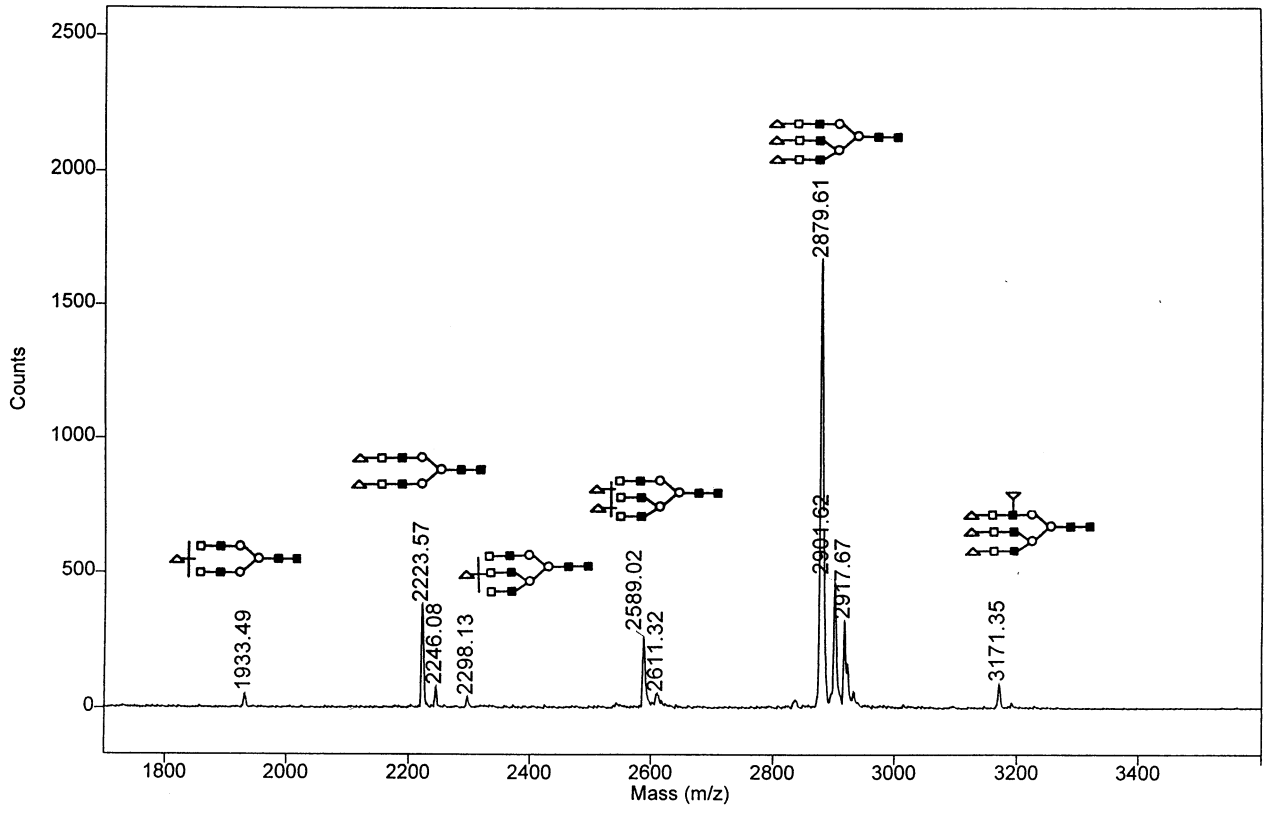

Figure 5. Negative-ion spectra of the N-linked oligosaccharides derived from the equivalent to 1 μg bovine fetuin. Symbols as in Figure 3.

matrix, application to the actual glycoprotein analysis has further been desirable. As demonstrated below, the DHB/spermine matrix (unlike a previously used THAP matrix [7]) is effective in the analysis of glycoprotein hydrolyzates without an essential need for desalting procedure. Understandably, additional desalting (drop dialysis [12]) improves the detection performance (Figure 4a vs. b), as shown with the example of human AGP N-glycans after their enzymatic release. Although a minor degree of fragmentation is observed in both cases (Figure 4a, b), the major signals are easily implicating the structures known to exist [23] in human AGP. The spectrum illustrated in Figure 4a, b does not show signals for some N-glycans structures that are known to exist in AGP (i.e., tetraantennary and fucosylated tetraantennary) [23]; however, the lack of signals for these structures is not because of the inability of the matrix to ionize larger structures, but rather to the fact that these structures were not cleaved from the glycoprotein. With the procedure used here, the two disulfide bonds existing in AGP were not cleaved prior to deglycosylation, causing incomplete deglycosylation. This was supported by a complete agreement between the spectral profiles observed with the sialylated oligosaccharides and their corresponding desialylated structures. When no desalting is performed (Figure 4a), the major mass-to-charge ratio values correspond to the sodium adducts: 2245, 2923, and 3069, corresponding to $[M+Na-2H]^-$, $[M+2Na-3H]^-$, and $[M+2Na-3H]^-$, respectively. (Note that the number of sodium adducts is equivalent to the number of acidic groups minus one.) Although monosodium adduction was observed in the case of trisialylated structures (Figure 4a), the signal intensities were weaker than for the disodiated species. The obtained sensitivity is genuinely high: the signals observed in Figure 4 reflect the N-glycans released from just 1.0 μg of human AGP, as determined from 2 mg/mL concentration of the N-glycan stock solution. N-Glycans released from bovine fetuin, one of the most documented glycoproteins, are shown in Figure 5. The major signals observed at m/z 1933, 2223, 2298, 2589, 2879, and 3171 correspond to $[M-H]^-$ ions of the monosialylated biantennary, disialylated biantennary, trisialylated triantennary, and tetrasialylated triantennary structures, respectively. These are the N-glycan structures known to exist [24] in fetuin, whereas the intensities of their respective signals reflect well the percentages in which they are commonly encountered. No extensive fragmentation of these parent structures was observed. Once again, we observed that desalting enhanced peak intensities with bovine fetuin as well (data not shown).

Because of its capability to analyze intact sialylated glycans, the DHB/spermine matrix in conjunction with the utility of specific neuraminidases and the subsequent MALDI/MS analysis can also be effectively used

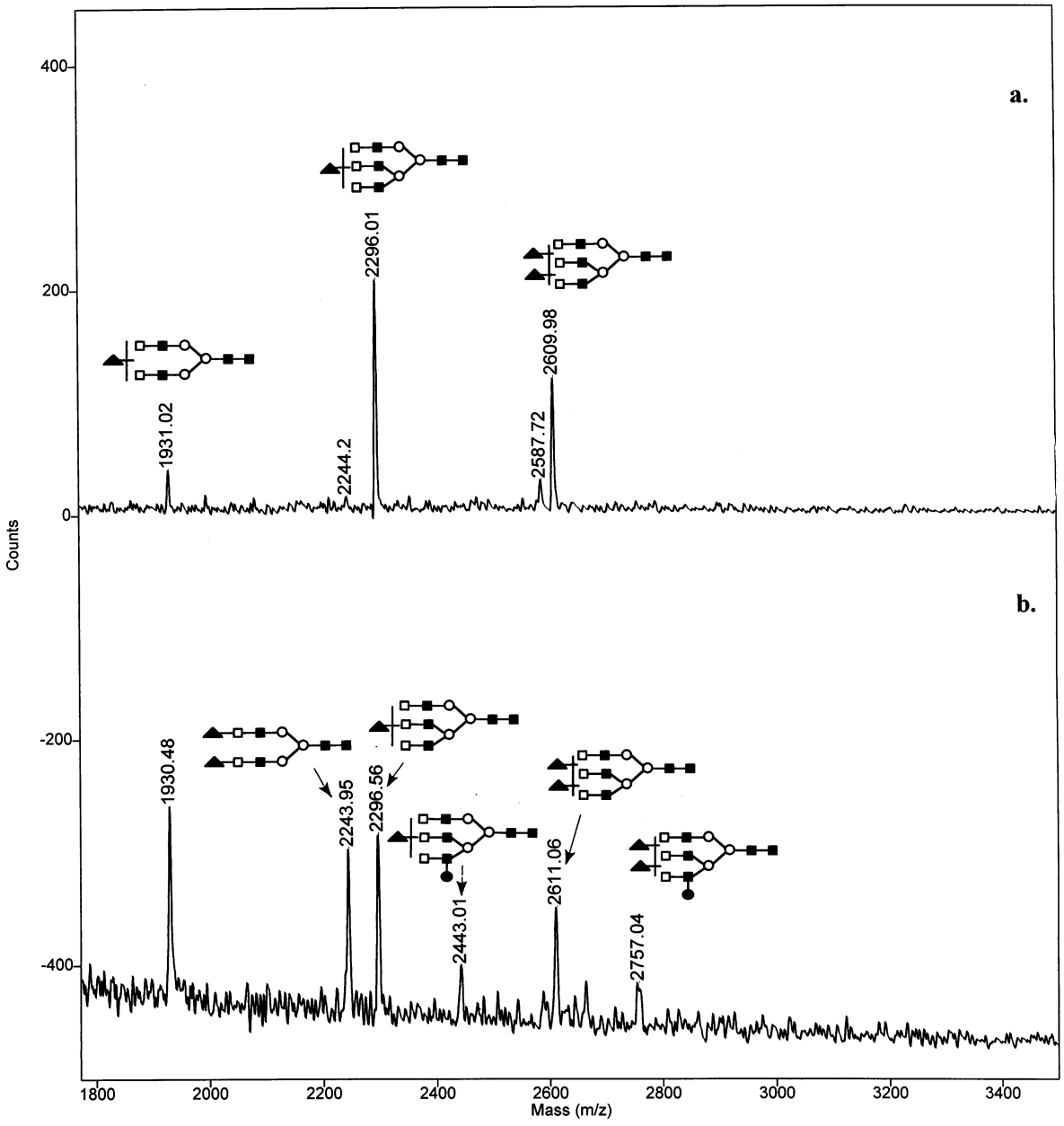

Figure 6. Negative-ion spectra of the N-linked oligosaccharides from bovine fetuin (**a**) and human AGP (**b**) treated with neuraminidase from Newcastle disease virus. Symbols as in Figure 3, and filled triangle = N-acetylneuraminic acid α(2–6).

to probe the sialic acid linkages existing in acidic structures. As an example, the negative-ion spectra of the acidic oligosaccharides isolated from bovine fetuin and human AGP are shown (Figure 6a, b) after the glycans were treated with neuraminidase from Newcastle disease virus. This enzyme is known to hydrolyze specifically terminal α(2–3)-bound sialic acids of either N- or O-linked glycans. The enzymatic treatment of the N-glycans from bovine fetuin caused disappearance of some signals and enhancement of native glycans (compare Figures 5 and 6a). The signals recorded after treatment with this neuraminidase corresponded to a disialylated triantennary (m/z 2609), monosialylated triantennary (m/z 2296), and monosialylated biantennary (m/z 1931) structures. A very weak signal corresponding to a disialylated biantennary structure (m/z 2244) was also observed. It can thus be concluded that none of the tri- or tetrasialylated structures have the sialic acid groups linked solely via α(2–6) linkage (no signals at m/z 2879 or 3171 were observed). In comparison, the positive-ion spectra (data not shown) of N-glycans from the same origin (after treatment with the same neuraminidase) exhibited signals at mass-to-charge ratio values corresponding to the biantennary and triantennary structures, suggesting the sialic acid attachments of the α(2–3) type. Although this method

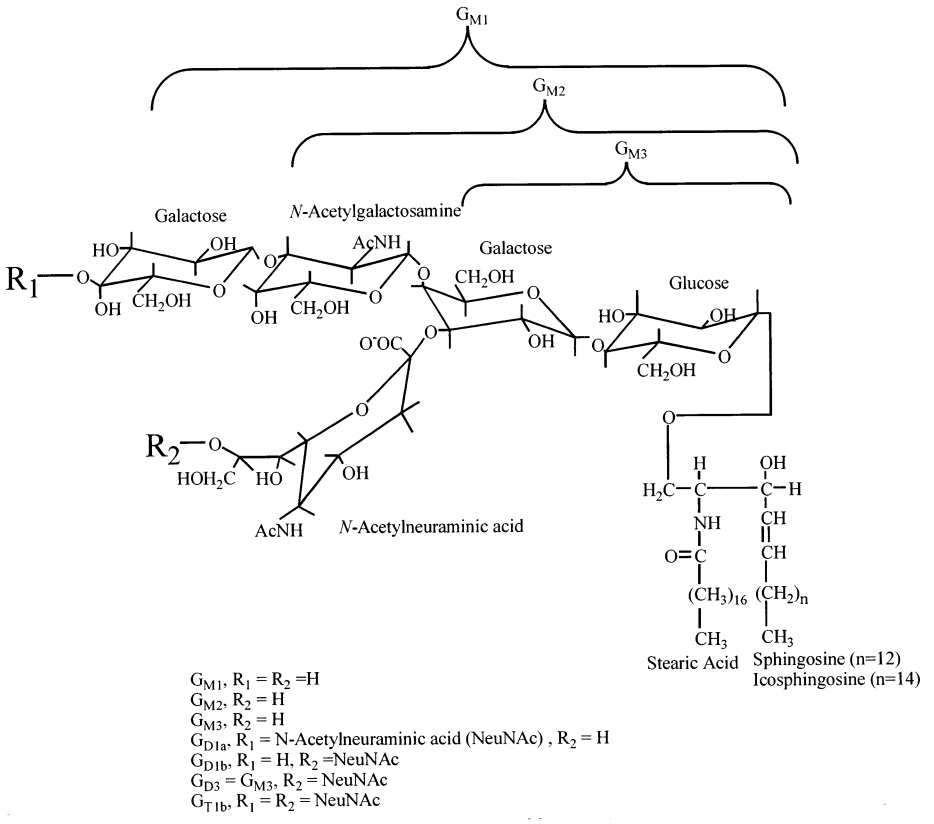

Diagram 1

allowed determination of the sialic acid linkages existing in sialylated oligosaccharides, it cannot provide the information on a specific antenna on which such linkages exist.

The negative-ion MALDI spectrum of the N-glycans from human AGP differed, after treatment with neuraminidase from Newcastle disease virus, from the original (untreated) sample (compare Figures 4 and 6b). The mass-to-charge ratio values determined from the negative-ion spectrum corresponded to a monosialylated biantennary (m/z 1930), disialylated biantennary (m/z 2244), monosialylated triantennary (m/z 2297), monosialylated/fucosylated triantennary (m/z 2443), disialylated triantennary (m/z 2611), and disialylated/fucosylated triantennary (m/z 2757) structures. These negative-ion spectra suggest the presence of structures featuring a mixture of sialic acid groups that are both α(2–3) and α(2–6) linked. In addition, a positive-ion MALDI spectrum of AGP N-glycans treated with neuraminidase (results not shown) exhibited signals corresponding to biantennary, triantennary, and fucosylated triantennary structures, thus suggesting the presence of structures that possesses sialic acid residues solely as α(2–3) linked. Thus, combining the data from both the negative- and positive-ion spectra with the enzymatic selectivity should reveal the true heterogeneity of sialic-acid linkages associated with N-glycans.

Because the combined DHB/spermine matrix proved effective for the acquisition of negative-ion spectra with acidic oligosaccharides, an extension to gangliosides (which also feature sialic residues) appeared natural. Gangliosides are glycosphingolipids consisting of a hydrophilic sialooligosaccharide chain and a hydrophobic ceramide moiety which contains a sphingosine and a fatty acid. A type IV ganglioside mixture essentially contains G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} (see Diagram 1).

In agreement with these structures, a negative-ion spectrum acquired for these gangliosides (Figure 7a) features three major regions because of various mixture constituents. The intensities of the signals observed reflect the natural concentration at which each of these gangliosides exist in a type IV mixture [25]. We note that each ganglioside type is responsible for two signals that differs by 28 m/z and correspond to sphingenine (d18:1) and icosasphingenine (d20:1) for each structure. The ceramide nomenclature depicted between the brackets indicate the number of hydroxy groups (e.g., ″d′ for dihydroxy), the number of carbon atoms, and the degree of saturation, respectively. The additional multiple signals observed in Figure 7a are not because of a multiple adduct formation, but rather because of the lactones formed between the sialic acid groups and the preceding galactose residues [25]. Fortunately, these lactones can be eliminated by treatment with ammonium hydroxide, as depicted in Figure 7b. As a result of this treatment, the negative-ion spectra have been simplified. In Figure 7b, the signals observed at m/z 1385

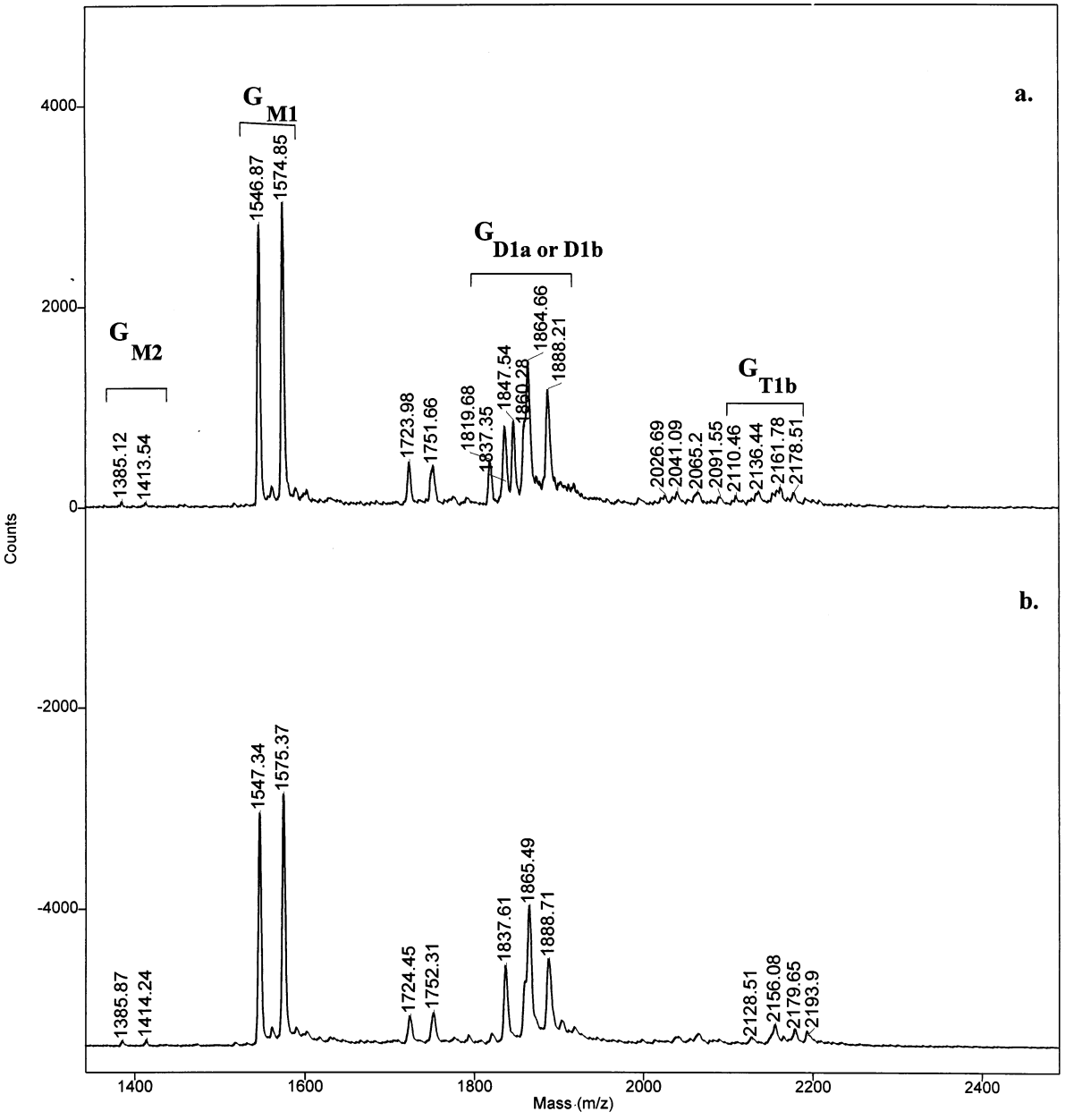

Figure 7. Negative-ion spectra of a ganglioside mixture before (**a**) and after (**b**) treatment with ammonium hydroxide.

and 1414 correspond to $[M - H]^-$ ions of the G_{M2} structure, whereas m/z 1547 and 1575 correspond to $[M - H]^-$ ions of the G_{M1}. Signals at m/z 1837 and 1865 correspond to $[M - H]^-$ ions of $G_{D1a\ or\ 1b}$ having sphingosine (d18:1) and icosasphingosine (d20:1), respectively, whereas $[M+Na - 2H]^-$ ions of $GD_{1a\ or\ 1b}$ were determined at m/z 1859 and 1888, respectively. Finally, the signals at m/z 2128 and 2156 correspond to G_{T1b} structure.

Conclusions

Inclusion of spermine as a co-matrix to DHB has been shown here to improve substantially the scope of the MALDI analysis for acidic glycans. Simple mass spectra were successfully acquired in the negative-ion mode for a variety of sialylated oligosaccharides and gangliosides down to the femtomole levels. Addition of spermine to DHB appears to minimize sodium adduction, thus reducing the needs for sample desalting prior to a MALDI analysis. In the cases when spermine does not totally eliminate adduct formation, the major ion produced during the MALDI process is easily accounted for as an equivalent to the number of acidic groups minus one sialic residue. Through the use of specific neuraminidases, the nature of sialic linkages can also be assessed.

Acknowledgment

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